Fluorogenic hydrogen sulfide (H₂S) donors based on sulphenyl thiocarbonates enable H₂S tracking and quantification†

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Hydrogen sulfide (H₂S) is an important gaseous molecule that plays critical roles in living systems. Endogenous H₂S is primarily produced from cysteine (Cys) and homocysteine (Hcy) by four main enzymes, including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 3-mercaptoppyruvate sulfur transferase (3-MST), and cysteine aminotransferase (CAT).  

Due to its bioregulatory and protective roles, H₂S is considered as an important cellular signaling molecule, much like nitric oxide (NO) and carbon monoxide (CO). Although many H₂S-related biological functions have been discovered in the past two decades, many investigations have been limited due to the lack of controllable and refined H₂S delivery systems (H₂S donors). Inorganic sulfide salts, such as sodium sulfide (Na₂S) and sodium hydrosulfide (NaHS), are widely used in H₂S investigations, but they release H₂S quickly in aqueous media, thus failing to mimic the slow and well-regulated in vivo H₂S generation. By contrast, the H₂S release from GYY4137, a phosphorodithioate-based H₂S donor, is slow in aqueous buffer, and the low H₂S releasing efficiency remains as a major concern when applying it to the living systems. Building from these donor scaffolds, a series of synthetic H₂S donors have been developed in the last decade.  

These donors are activated by different triggers, such as enzymes, cellular thiols, pH modulation, and photo activation, and the released H₂S exhibits promising activities in different physiological and pathological processes.  

To expand the library of H₂S donors, we recently developed the carbonyl sulfide (COS)-based platform for H₂S donation. In this approach, caged-thiocarbamates are activated and the resultant intermediates undergo a cascade reaction to release COS, which is quickly converted to H₂S by the ubiquitous enzyme carbonyl anhydrase (CA) (Fig. 1a). Expanding on this initial report, we, as well as others, have applied the similar caged-COS systems to include a series of triggerable COS-based H₂S donors which can be activated by different mechanisms, such as reactive oxygen species (ROS), esterase, cellular nucleophiles, click chemistry, light, and Cys, to release H₂S under various conditions.  

Although H₂S release from these donors has been measured using different methods, the real-time tracking of donor activation and H₂S delivery in living systems remains a key challenge due to the inherent limitations of current H₂S detection
results and discussion

To test our hypothesis that caged-sulfenyl thio-carbonates could serve as thiol-triggered fluorescent COS/H_2S donors, four donors (FLD-1–4) were prepared by reacting fluorescein or 3-O-methylfluorescein with ((benzyl)dithio)carbonyl chloride 1 or ((phenol)dithio)carbonyl chloride 2 in the presence of DIPEA (Scheme 1a). A caged thiocarbonate (TCN-1) was synthesized as a triggerless control compound, which is expected to be stable toward thiol activation due to the lack of a disulfide functional group (Scheme 1b).

With these compounds in hand, we first evaluated their spectroscopic properties in PBS buffer (pH 7.4, 10 mM). As expected, FLD-1–3 and TCN-1 are not absorbive in the visible region and are all nonfluorescent because the fluorescein unit is locked in the closed lactone form. By contrast, FLD-4 shows a prominent absorbance band in the visible region ($\lambda_{\text{max}} = 449$ nm, $\epsilon = 27.300 \pm 2500$ M$^{-1}$ cm$^{-1}$) with measurable fluorescence ($\lambda_{\text{em}} = 514$ nm, $\Phi = 0.11 \pm 0.01$) due to the free hydroxyl group (Table S1†). Based on the promising spectroscopic properties, large dynamic range, and efficient release of two equivalents of COS/H_2S, we chose to use FLD-1 as the model donor for initial reactivity and selectivity evaluations.

To test the reactivity of FLD-1 towards Cys-induced activation, FLD-1 (10 $\mu$M) was incubated with Cys (100 $\mu$M) in PBS buffer (pH 7.4, 10 mM) containing physiologically-relevant concentrations of CA (25 $\mu$g mL$^{-1}$), and the fluorescence intensity was measured using a fluorescence spectrometer. As expected, Cys successfully activated FLD-1 and resulted in a 500-fold fluorescence turn on over 2 h, demonstrating the release of the fluorescein upon FLD-1 activation (Fig. 2a). Fluorescein formation was also confirmed by UV-vis spectroscopy under the identical conditions (Fig. S1†). A Cys-dependent fluorescence enhancement was observed when treating FLD-1 (10 $\mu$M) with increasing concentrations of Cys (1–20 equiv.), indicating a high sensitivity of FLD-1 towards Cys. No fluorescent signal was observed in the absence of Cys, suggesting that FLD-1 is stable in aqueous buffer, and that it is not hydrolyzed to provide false
signals (Fig. 2b). In addition, to confirm the H$_2$S delivery from FLD-1, we treated FLD-1 (10 μM) with Cys (100 μM) in PBS containing CA (25 μg mL$^{-1}$) and quantified H$_2$S release using the MB assay. In this experiment, 15 μM of H$_2$S was detected (75% releasing efficiency), which is consistent with our hypothesis that 2 equivalents of COS/H$_2$S would be released upon FLD-1 activation (Fig. S2†). Taken together, these experiments demonstrate that FLD-1 is highly responsive to Cys activation.

To determine whether H$_2$S release correlated directly with the observed fluorescence response, we measured the fluorescent response from FLD-3 in the presence of Cys and CA and quantified H$_2$S release using the MB assay. We chose to use FLD-3 as the model compound for these investigations because it only contains one sulfenyl thiocarbonate moiety, and therefore should simplify the reaction kinetics. In comparison, FLD-1 contains two sulfenyl thiocarbonate groups, and the cleavage of one sulfenyl thiocarbonate would generate FLD-4 as a reaction intermediate, which exhibits moderate fluorescence (see Fig. 4). Incubation of FLD-3 (10 μM) with Cys (100 μM) resulted in a rapid fluorescence response with 96% of the H$_2$S release measured by MB. At extended time points, we observed a slight decrease in measured H$_2$S, possibly due to volatilization of H$_2$S determined by MB. At extended time points, we observed a slight decrease in measured H$_2$S, possibly due to volatilization of H$_2$S in the headspace of the closed system or adventitious oxidation of released H$_2$S. Negligible H$_2$S was detected in the absence of CA, indicating that Cys-triggered H$_2$S delivery from FLD-3 proceeds through intermediate COS formation (Fig. 3a). Importantly, the strong linear correlation between the measured fluorescence and H$_2$S measured from the MB method detection (first 25 min, $R^2 = 0.988$) demonstrates that fluorescent readouts can serve as reliable optical tools to track COS/H$_2$S release from FLD donors with temporal resolution (Fig. 3b). Moreover, this linear correlation suggests that choice of other fluorophores with different brightnesses and photophysical properties could be used to access different dynamic ranges of H$_2$S release, thus enabling this approach to be translated to different types of experimental designs.

Having confirmed the efficiency of Cys-triggered fluorescence turn on and COS/H$_2$S release, we next evaluated the reactivity of other FLD donors towards Cys activation. Treating FLD-1–3 (10 μM) with Cys (100 μM) in PBS buffer (pH 7.4, 10 mM) resulted in a 120–500-fold fluorescence turn on over 2 h. We attribute the faster response of FLD-2 to the more electrophilic phenyl sulfenyl thiocarbonate in comparison to the less electrophilic benzyl sulfenyl thiocarbonate in FLD-1. FLD-4, however, provided minimal fluorescence enhancement due to its strong background fluorescence. No fluorescence response from TCN-1 (10 μM) was observed under the identical conditions, which demonstrates the stability of the thiocarbonate group in the presence of Cys (Fig. 4).

To further support our proposed activation mechanism in Fig. 1b, as well as the activation of the sulfenyl thiocarbonate group by other thiols, we incubated FLD-1 (10 μM) in PBS...
(pH 7.4, 10 mM) with 10 equivalents of benzyl mercaptan (100 μM) for 1 h and analyzed the reaction products by HPLC (Fig. S3†). Consistent with our proposed activation mechanism, we observed FLD-1 consumption and the formation of both benzyl disulfide and fluorescein, which supports the proposed mechanism.

In addition to Cys and BnSH, other cellular thiols and biological nucleophiles were tested to determine whether they resulted in donor activation. In these experiments, FLD-1 (10 μM) was incubated with GSH, homocysteine (Hcy), N-acetyl cysteine (NAC), penicillamine (PEN), or bovine serum albumin (BSA) (100 μM), in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg mL⁻¹) and the fluorescent intensity was measured after 2 h. As expected, FLD-1 is stable in PBS at physiological pH in the absence of thiols (Fig. 5 bar 1). Incubation of FLD-1 with Cys, NAC, GSH, and Hcy, however, led to a significant fluorescence enhancement, indicating successful donor activation and COS/H₂S release (Fig. 5 bars 2–5 and S4†). In addition, these results demonstrate that the sulfenyl thiocarbonate group is responsive to different types of thiols. In comparison, PEN resulted in only minimal fluorescence response and BSA failed to activate the donor presumably due to the bulkiness of these two thiol species, which hindered their reactions with FLD-1 (Fig. 5 bars 6 and 7). Additionally, N-ethylmaleimide (NEM) pretreatment of Cys samples significantly reduced the fluorescence enhancement from FLD-1, confirming the necessity of the thiol-induced reduction for the donor activation (Fig. 5 bar 8).

We also tested the response of the FLD donors in the presence of other cellular reactive sulfur, oxygen, and nitrogen species (RSONs). FLD-1 (10 μM) was incubated with RSONs (100 μM), such as hydrogen peroxide (H₂O₂), hyperchlorite (ClO⁻), superoxide (O₂⁻), tert-butyl hydroperoxide (TBHP), serine (Ser), lysine (Lys), glycine (Gly), oxidized glutathione (GSSG), and S-nitrosoglutathione (GSNO), and COS/H₂S release was monitored using C7-Az, a H₂S-responsive fluorescent probe.⁵⁰,⁵¹ Incubation of HeLa cells with C7-Az (50 μM) alone resulted in a negligible fluorescence response, indicating minimal endogenous H₂S (Fig. 6 top row). Treatment with TCN-1 also failed to provide a fluorescence signal, suggesting that the thiocarbonate group was stable and did not decompose to generate false signals in cellular environments (Fig. 6 middle row). By contrast, a strong C7-Az fluorescent signal was observed when incubating HeLa cells with FLD-1, suggesting that H₂S release was successfully triggered by endogenous thiols. In addition, a strong fluorescence signal was also observed from activated FLD-1 in the fluorescein channel, confirming the fluorescence response upon donor activation (Fig. 6 bottom row). Taken together, these results demonstrate that the FLD donors not only function as efficacious H₂S donors in live cells, but also provide a fluorescence signal that enables observing H₂S release.

To further demonstrate the H₂S-releasing fidelity of the FLD donors we also investigated the anti-inflammatory activities of FLD-1. We pretreated macrophage RAW 264.7 cells with FLD-1 (0–25 μM) for 2 h, followed by a 24 h incubation with lipopolysaccharide (LPS, 0.5 μg mL⁻¹) to trigger the inflammatory...
response. The inflammation event usually results in the NO generation, which can be monitored by measuring nitrite (NO$_2^-$) accumulation. We chose to use concentrations of FLD-1 up to 25 μM because these concentrations did not induce cytotoxicity (Fig. S6†).

As expected, the pretreatment of RAW 264.7 cells with FLD-1 showed a dose-dependent inhibition of NO$_2^-$ accumulation, indicating anti-inflammatory activity from FLD-1. Although GYY4137 has shown anti-inflammatory effects at higher concentration and longer incubation time (i.e. 100–1000 μM and 24 h incubation), such cytoprotection was not observed at the 25 μM concentration used for comparison, highlighting the efficacious H$_2$S release from FLD-1 in the cellular environment (Fig. 7). To further confirm that the observed effects were due to H$_2$S rather than other components of donor activation, we treated cells with 25 μM of TCN-1, fluorescein or benzyl mercaptan and measured NO$_2^-$ production. None of these compounds attenuated NO$_2^-$ generation, confirming that the anti-inflammatory activities of FLD-1 is due to H$_2$S release (Fig. S7†). Overall, these studies demonstrate that FLD-1 releases COS/H$_2$S in complex cellular environment and exhibits promising anti-inflammatory protections, indicating potential applications of FLD-1 as H$_2$S-releasing therapeutics.

Conclusions

In conclusion, we prepared and evaluated a class of caged sulfonyl thiocarbonates as new controllable and fluorescent COS/H$_2$S donors. Thiol-triggered COS/H$_2$S release from these molecules has been detected and visualized in both aqueous buffer and in live cells. Importantly, the concomitant release of a fluorescein reporter after H$_2$S release enables the real-time monitoring of H$_2$S release dynamics. In addition, we demonstrate that FLD-1 exhibits a dose-dependent inhibition of the LPS-induced NO formation, which is consistent with anti-inflammatory activities of H$_2$S. Taken together, caged-sulfonyl thiocarbonates are promising new class of COS/H$_2$S donors with high potential for accessing H$_2$S-related protective activities, and the developed fluorescent donors provide new methods for monitoring H$_2$S release in real-time in complex environments.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

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